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# Lipoprotein(a) binds to fibronectin and has serine proteinase activity capable of cleaving it

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**The plasma concentration of human lipoprotein(a) [Lp(a)] is correlated with the risk of heart disease. A distinct feature of the Lp(a) particle is the apolipoprotein (a) [apo(a)], which is associated with apoB-100, the main protein component of low-density lipoprotein. We now report that apo(a), which has extensive homology to plasminogen, binds to immobilized fibronectin. The binding of Lp(a) was localized to the C-terminal heparin-binding domain of fibronectin. Incubation of Lp(a) with fibronectin resulted in fragmentation of fibronectin. The cleavage pattern, as visualized by gel electrophoresis and immunoblotting, was reproducibly obtained with Lp(a) purified from five different individuals and was distinct from that obtained upon proteolysis of fibronectin by plasmin or kallikrein. The use of synthetic peptide substrates demonstrated that the amino acid specificity for Lp(a) was arginine rather than lysine. The proteolytic activity of Lp(a) was localized to apo(a) and experiments with inhibitors indicated that the proteolytic activity was of serine proteinase-type.**

*Key words:* atherosclerosis/fibronectin/lipoprotein(a)/plasminogen/serine proteinase

## Introduction

According to several recent studies, the plasma concentration of human lipoprotein(a) [Lp(a)] is correlated with the risk for atherosclerosis (Kostner *et al.*, 1981; Armstrong *et al.*, 1986; Rhoads *et al.*, 1986). Lp(a) has a lipid composition similar to that of low-density lipoprotein (LDL) (Simons *et al.*, 1970) but, in addition to the apoB-100 characteristic of LDL, Lp(a) contains apoprotein(a) [apo(a)] (Ehnholm *et al.*, 1972; Utermann and Weber, 1983), a highly glycosylated protein which displays genetically determined size heterogeneity (Utermann *et al.*, 1987). Apo(a) can be dissociated from apoB-100 under reducing conditions (Gaubatz *et al.*, 1983; Seman and Breckenridge, 1986). The molecular mass of apo(a) can range from 300 000 to ~800 000 daltons (Kratzin *et al.*, 1987).

Recently, the sequences for human and rhesus monkey apo(a) have been determined from cDNA clones (McLean *et al.*, 1987; Tomlinson *et al.*, 1989). The results showed that apo(a) has a striking homology to plasminogen as well as remarkable internal repeated sequences. Human apo(a) contains 37 repeats of a sequence that is highly homologous to the kringle 4 domain of human plasminogen. Also the proteinase domains of human apo(a) and plasminogen share extensive homology, and the catalytic triad (Ser, His, Asp), and the cysteine residues of plasminogen are retained (McLean *et al.*, 1987). However, at the site where plasminogen is cleaved by its activators to produce active plasmin, the arginine residue has been replaced by serine in apo(a), a substitution that has suggested that apo(a) may not be converted to an active serine proteinase. Also studies with purified apo(a) or Lp(a), either alone or in combination with tissue plasminogen activator (t-PA), urokinase or streptokinase, have failed to demonstrate plasmin-like activity (Eaton *et al.*, 1987; McLean *et al.*, 1987). However, there is an early report (Jürgens *et al.*, 1977) in which amidolytic activity against artificial substrates *N*-α-tosyl-L-arginine-methylester–HCl (L-TAME) and *N*-α-benzoyl-L-arginine-4-nitroanilide–HCl (L-BAPA) was found to be associated with isolated apo(a).

The plasma concentration of Lp(a) shows genetically determined variation (Harvie and Schultz, 1970), which is linked to the locus of the apo(a) gene (Frank *et al.*, 1988; Lindahl *et al.*, 1989). The plasma level of Lp(a) is not correlated with other lipid risk factors such as apolipoproteins A-I and B, LDL-cholesterol or HDL-cholesterol (Rhoads *et al.*, 1986). Therefore, the hypothesis that the cholesterol-rich Lp(a) particle may be atherogenic by binding to sites of the vessel wall where there is endothelial cell damage, via fibrin or other molecules, is an attractive one.

Fibronectin is a high mol. wt glycoprotein present in plasma in a soluble form and in connective tissue matrices in an insoluble form (Vaheri *et al.*, 1985; Mosher, 1989). Fibronectin is known to be sensitive to several proteinases, including plasmin, and the degradation of fibronectin results in loss of the integrity of the cellular matrix (Vartio *et al.*, 1981; Vaheri *et al.*, 1985). Further characteristics of fibronectin are its multiple interactions with other proteins including fibrin, collagen (Mosher, 1989) and components of the coagulation system such as plasminogen and its activators (Salonen *et al.*, 1985).

It has been demonstrated that fibronectin is an early marker of connective tissue formation and that increased levels of fibronectin, fibrinogen and fibrin together with Lp(a) are present in early atherosclerotic lesions and in atherosclerotic plaques (Walton *et al.*, 1974; Stenman *et al.*, 1980; Vaheri *et al.*, 1983).

To find out whether interactions occur between the components of atherosclerotic lesions and Lp(a), we studied the binding of Lp(a) to fibronectin. We now provide evidence that Lp(a) and apo(a) bind to fibronectin and that this interaction leads to proteolytic cleavage of fibronectin. The use of inhibitors suggests that the proteolytic activity of apo(a) is that of a serine proteinase.

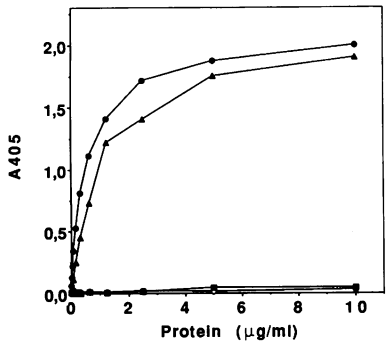


**Fig. 1.** Binding of Lp(a) and apo(a) to immobilized fibronectin. Purified fibronectin or albumin (4 μg/ml) was immobilized onto polystyrene wells. Increasing concentrations of Lp(a), apo(a) and LDL, in 75 μl of 0.01 M sodium phosphate buffered 0.15 M NaCl, pH 7.4, containing 0.02% (v/v) Tween 20 and 4% (w/v) polyethylene glycol 6000, was added and incubated at room temperature for 2 h. After washing twice with 200 μl of distilled water the fibronectin-bound Lp(a) or apo(a) was determined by adding 75 μl of monospecific polyclonal rabbit anti-Lp(a) IgG (2.5 μg protein/ml) and incubated for 1 h at room temperature. After washing, the wells were incubated for 1 h at room temperature with a 1:100 dilution of alkaline phosphatase-labelled antibodies to rabbit IgG. After washing, the binding of labelled antibodies was visualized using 0.2% (w/v) disodium *p*-nitrophenylphosphate as a substrate. The reaction was stopped with 1 M NaOH and the amount of *p*-nitrophenol formed determined spectrophotometrically at 405 nm. LDL was determined similarly but using monospecific anti-LDL. ●———● Lp(a) bound to immobilized fibronectin; ▲———▲ apo(a) bound to immobilized fibronectin; ■———■ LDL bound to immobilized fibronectin; ○———○ Lp(a) bound to immobilized albumin.

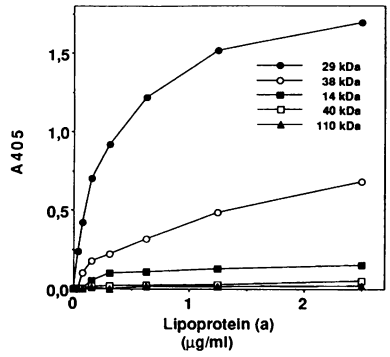


**Fig. 2.** Binding of Lp(a) to immobilized thermolysin fragments of fibronectin. The purified 14, 29, 38, 40 and 110 kd thermolysin fragments of plasma fibronectin (2 μg/ml) were immobilized onto polystyrene wells. Increasing amounts of Lp(a) were then added to the wells and processed as described in the legend to Figure 1. Fragments used for coating: ■———■ 14 kd; ●———● 29 kd; ○———○ 38 kd; □———□ 40 kd; ▲———▲ 110 kd.

## Results

### *Binding of Lp(a) to immobilized fibronectin and its fragments*

To study the interaction between human Lp(a) and fibronectin, fibronectin was immobilized to polystyrene and incubated with increasing concentrations of purified Lp(a). Figure 1 illustrates one such experiment and demonstrates that Lp(a) binds to immobilized fibronectin in a concentration-dependent and saturable manner. Binding of Lp(a) to fibronectin could be demonstrated with this assay when the concentration of Lp(a) used in the incubation was 20 ng/ml or higher. LDL ($1.02 < d < 1.05$ g/ml) did not bind to immobilized fibronectin and the addition of increasing

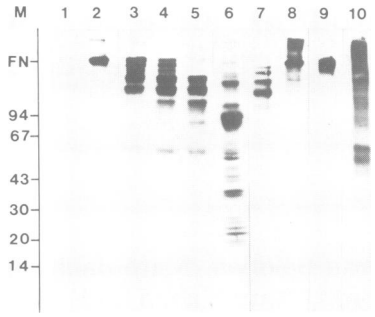


**Fig. 3.** Cleavage of fibronectin by Lp(a). The indicated amounts of proteins were incubated for 72 h at 37°C in an end-over-end mixer. The mixtures were then analysed under reducing conditions by SDS–PAGE followed by immunoblotting with antibodies to human fibronectin. **Lane 1**, Lp(a) 2.5 μg; **lane 2**, fibronectin 2.5 μg **lane 3**, Lp(a) 0.6 μg + fibronectin 2.5 μg; **lane 4**, Lp(a) 1.25 μg + fibronectin 2.5 μg; **lane 5**, Lp(a) 2.5 μg + fibronectin 2.5 μg; **lane 6**, plasmin 1 μg + fibronectin 2.5 μg; **lane 7**, kallikrein 20 μg + fibronectin 2.5 μg; **lane 8**, Lp(a) 2.5 μg + fibronectin 2.5 μg + PMSF 20 mM; **lane 9**, LDL 5 μg + 2.5 μg fibronectin; **lane 10**, apo(a) 2.5 μg + 2.5 μg fibronectin. The LDL used had been purified by ultracentrifugation and gel filtration (fraction 52, Figure 4). The mol. wts of the marker proteins (M) are shown in kd.

amounts of LDL (up to 20 μg protein/ml) did not affect the binding of Lp(a) to fibronectin, suggesting that the binding of Lp(a) was due to apo(a). Similar results were obtained using Lp(a) purified from five different subjects. Human high-density lipoprotein (HDL) ($1.12 < d < 1.21$ g/ml) did not bind to immobilized fibronectin. To further characterize the interaction we used purified apo(a). This was isolated from Lp(a), treated with dithioerythritol, by gradient ultracentrifugation. The isolated apo(a) bound to fibronectin in a similar manner as Lp(a) (Figure 1). In experiments in which Lp(a) was immobilized on polystyrene and soluble fibronectin was added to the incubation only marginal binding of fibronectin could be observed.

To further characterize the interaction between apo(a) and fibronectin several compounds were tested for possible interference. None of the compounds tested, Arg-Gly-Asp peptide (10 μM), L-arginine (5 μM), heparin (20 IU/ml), aprotinin (200 IU/ml), $CaCl_2$ (10 mM), $MgCl_2$ (10 mM) and ε-aminocaproic acid (100 mM) interfered with the binding of Lp(a) to fibronectin.

To define which region of fibronectin Lp(a) binds to thermolysin fragments of human plasma fibronectin, isolated by hydroxyapatite chromatography, were used. These included the 40 kd gelatin-binding fragment, a 14 kd central heparin-binding fragment, a 110 kd cell-binding fragment and overlapping carboxy-terminal heparin-binding 29 and 38 kd fragments, which were immobilized and tested for Lp(a) binding. Of these only the carboxy-terminal heparin-binding fragments showed significant binding (Figure 2).

### *Proteolytic activity*

The interaction of purified Lp(a) with fibronectin resulted in proteolytic cleavage of fibronectin. When fibronectin was incubated with Lp(a) or apo(a), a distinct cleavage pattern of fibronectin could be observed upon SDS–PAGE (Figure 3). The proteolysis resulted in fragments with apparent mol. wts of 180, 160, 130, 115 and 63 kd. This cleavage pattern, which was distinct from that caused by plasmin, kallikrein (Figure 3), cathepsin G (Vartio *et al.*, 1981) or thermolysin (Zardi *et al.*, 1985), was reproducibly

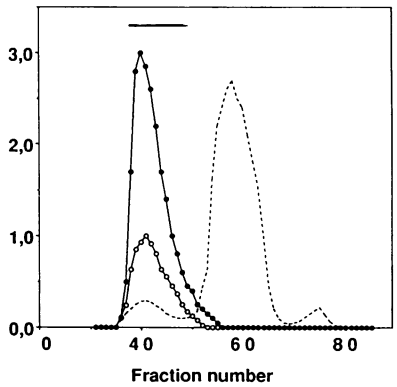


**Fig. 4.** Gel filtration chromatography of human Lp(a). The Sepharose 4B column (2.5 × 100 cm) was equilibrated with phosphate-buffered saline, pH 7.4, and the density fraction $1.05 < d < 1.12$ g/ml was chromatographed at a flow rate of 28 ml/h. The fractions were analysed for $A_{280}$ (----), Lp(a) concentration (mg/ml) (●———●) and Lp(a) amidolytic activity (○———○) (μmol/min × $10^{-1}$). For the enzyme activity measurements, 50 μl of the fractions was preincubated in 100 mM Tris−HCl buffer, pH 8.0, for 5 min and thereafter 150 μl of 4 mM *N*-α-Cbo-D-Arg-Gly-L-Arg-pNA (S-2765) substrate was added. The liberation of *p*-nitroaniline was recorded at $OD_{405}$. The activity is expressed as μmol *p*-nitroaniline liberated/min. The fractions that caused the degradation of fibronectin are indicated by a bar.

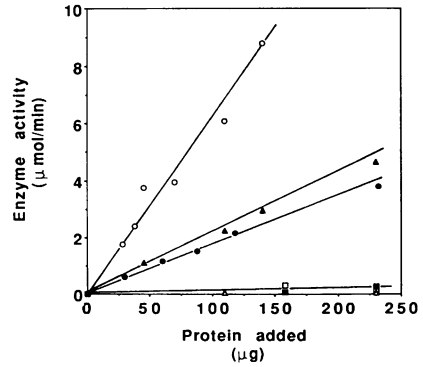


**Fig. 5.** Hydrolysis of chromogenic peptide substrates as a function of Lp(a), apo(a) and LDL concentration. Increasing amounts of Lp(a), apo(a) or LDL were pre-incubated in 100 mM Tris−HCl buffer, pH 8.0, for 5 min at 25°C. After pre-incubation, 200 μl of 4 mM substrate was added and the liberation of *p*-nitroaniline was recorded at $OD_{405}$. The amidolytic activity is expressed as μmol *p*-nitroaniline liberated/min. The activity of apo(a) (▲) and LDL (△) was measured using L-BAPA as substrate and the activity of Lp(a) with the substrates: ○———○ *N*-α-Cbo-D-Arg-Gly-L-Arg-pNA (S-2765); ●———● *N*-α-benzoyl-L-Arg-pNA (L-BAPA); □———□ D-Val-Phe-Lys-pNA (S-2390); ■———■ D-Val-Leu-Lys-pNA (S-2251).

**Table I.** Effect of inhibitors on Lp(a)-induced fragmentation of fibronectin[a]

| Lp[a] | FN | Inhibitor (concentration) | FN fragmentation |
|---|---|---|---|
| − | + | − | − |
| + | + | − | + |
| + | + | PMSF(10−20 mM) | − |
| + | + | Phenanthroline (10−20 mM) | + |
| + | + | Aprotinin (200 IU/ml) | + |
| + | + | NPGB (2−20 μg/ml) | + |
| + | + | EDAC (10−20 mM) | −[b] |

[a]For these experiments equal amounts (5 μg) of Lp(a) and fibronectin (FN) were incubated in NaCl/$P_i$ for 72 h in the presence or absence of the inhibitors, after which the reaction mixtures were analysed by SDS−PAGE and immunoblotting.
[b]Aggregation of fibronectin to high mol. wt complexes.

obtained with five different Lp(a) preparations originating from plasma of five different individuals. Digestion of fibronectin could be observed using Lp(a):fibronectin protein ratios ranging from 1:1 to 1:100. When the low ratio of Lp(a) was used, the incubation had to be prolonged for up to 72 h. To exclude the possibility that the proteolytic activity was due to contaminating proteins, sequential fractions from the last purification steps were tested for fibronectin cleaving activity. The distribution of proteolytic activity was found to be similar to that of Lp(a) in density gradient centrifugation (data not shown) as well as upon gel filtration (Figure 4). The fractions obtained from gel filtration were also tested for the presence of kallikrein by immunoblotting. No immunoreactive material could be detected. The specificity of the Lp(a) proteinase activity was studied using IgG, albumin, actin and vitronectin as controls. Incubation of these proteins with Lp(a) did not result in any proteolysis (data not shown).

As apo(a) shows extensive homology to plasminogen and has a similar proteinase region, it was of interest to study the effect of some known inhibitors on the Lp(a)-induced fragmentation of fibronectin. Table I summarizes the results obtained with different inhibitors. It is evident that the serine proteinase reagent, PMSF, inhibited fibronectin fragmentation (see also Figure 3). In addition, a carboxyl group-reagent, EDAC, also abolished the activity. This suggests that the proteolytic activity of Lp(a) is due to a serine proteinase-type activity. Antibodies to Lp(a) did not inhibit degradation of fibronectin.

As the proteolytic cleavage of fibronectin by Lp(a) is a rather slow process, we tested the effect of adding the plasminogen activators, t-PA and urokinase, to the incubation mixture. No effect of these activators could be observed on the proteolytic process.

The enzyme activity of Lp(a) was further characterized with the aid of chromogenic peptide substrates. Purified Lp(a) exhibited concentration-dependent amidolytic activity against *N*-α-benzoyl-L-arginine-pNA−HCl (L-BAPA), *N*-α-Cbo-D-arginine-glycine-L-arginine-pNA (S-2765) (Figure 5) and towards *N*-α-tosyl-L-arginine-methylester−HCl (L-TAME) (data not shown). However, even prolonged incubation of Lp(a) with the synthetic plasmin substrates D-valine-leucine-lysine-pNA (S-2251) and D-valine-phenylalanine-lysine-pNA (S-2390) did not result in hydrolysis of these substrates (Figure 5). Neither did it hydrolyse proline-phenylalanine-arginine-pNA (S-2302), a synthetic substrate considered specific for plasma kallikrein. Isolated apo(a), separated from Lp(a) under reducing conditions, also exhibited concentration-dependent amidolytic activity (Figure 5). In control experiments LDL, whether isolated from a Lp(a)-positive person (Figure 5) or from a subject with a Lp(a) null allele, did not hydrolyse any of the synthetic substrates.

Monitoring the gel filtration fractions obtained during Lp(a) purification showed that the amidolytic activity coincided with the Lp(a) concentrations of these fractions (Figure 4) while LDL was devoid of such activity. The inhibitors used in the studies with fibronectin and Lp(a) gave a similar inhibition pattern when tested using artificial substrates (data not shown). These experiments further corroborated our conclusion that Lp(a) has proteolytic

activity and that this enzyme activity belongs to the family of serine proteinases.

## Discussion

Our observations that Lp(a) binds to fibronectin and can cause degradation of the molecule are of great interest in the context that Lp(a) has been implicated as an independent risk factor in the development of atherosclerosis (Kostner *et al.*, 1981; Armstrong *et al.*, 1986; Rhoads *et al.*, 1986) and that fibronectin is one of the main protein components of early atherosclerotic lesions (Stenman *et al.*, 1980; Vaheri *et al.*, 1983).

The binding of Lp(a) to fibronectin is due to the apo(a) portion of Lp(a) as judged from competition studies employing LDL and from direct binding experiments with isolated apo(a). Disulphide bridge kringle structures present in prothrombin (Magnusson *et al.*, 1975), urokinase (Günzler *et al.*, 1982), tissue plasminogen activator (Pennica *et al.*, 1983) and fibronectin (Skorstensgaard *et al.*, 1982) are supposed to be involved in the binding of ligands essential for the regulation of the biological function of these proteins. As the main part of apo(a) consists of 37 kringle domains, similar to the kringle 4 region of plasminogen, this portion of the molecule is a likely candidate for fibronectin binding. Previous studies have demonstrated that plasminogen binds to immobilized fibronectin (Salonen *et al.*, 1985). The binding of plasminogen to fibronectin is localized to the 120–140 kd C-terminal fragment of the fibronectin molecule (Salonen *et al.*, 1985). We found that Lp(a) also binds to the C-terminal portion of the fibronectin molecule. This binding site could be localized to the 29 kd heparin-binding domain. The type of binding was, however, different from that of polyanions as heparin did not compete for Lp(a) binding. In addition to the 29 kd fragment, the 38 kd fragment also showed some binding activity. The 38 and 29 kd fragments are derived from the two fibronectin subunits which both contain three identical type III homologies. In addition, the 29 kd fragment contains part of the III CS domain, which has an internal thermolysin cleavage site, while the 38 kd contains a fourth type III homology (Zardi *et al.*, 1985; Pande *et al.*, 1987). This additional type III repeat in the 38 kd fragment is acidic which may affect its binding properties. Further studies are needed to define the exact sequences involved in the fibronectin–Lp(a) interaction.

The binding of apo(a) to fibronectin differed from that of plasminogen as it was not sensitive to $CaCl_2$ (up to 10 mM), a concentration known to inhibit plasminogen binding (Salonen *et al.*, 1985). The interaction also differed from that observed between plasminogen and fibrin in that the addition of ε-amino-carboxylic acids or lysine did not interfere with binding of Lp(a) to fibronectin and thus this interaction does not seem to depend on lysine-binding sites. Our experiments do not exclude binding of fibronectin to the kringle domain homologous to kringle 5 in plasminogen. As this part of the molecule is not present in the Lp(a) of rhesus monkey (Tomlinson *et al.*, 1989) it will be of interest to study the binding of Lp(a) from this animal to fibronectin.

The binding of Lp(a) to fibronectin resulted in a specific degradation of this molecule. The use of inhibitors enabled us to classify the proteolytic activity of Lp(a) as one due to a serine proteinase-type enzyme. The proteolytic activity is localized to the apo(a) portion of Lp(a). This conclusion is based on the following observations. The proteolytic activity co-migrated with Lp(a) during its purification from the plasma of five different individuals. The pattern of proteolysis caused by Lp(a) or apo(a) was clearly distinct from that caused either by plasmin or kallikrein. The enzyme activity was not affected by adding known plasminogen activators to the incubation system nor did it hydrolyse plasmin-specific or kallikrein-specific chromogenic substrates. In the Lp(a) preparations, no kallikrein could be detected by immunoblotting. Incubation of Lp(a) with added plasminogen did not result in plasmin formation as determined using immunoblotting, neither did Lp(a) function as a plasminogen activator. These results strongly suggest that Lp(a) has inherent proteolytic activity. These observations agree with those reporting no plasmin-like activity (Eaton *et al.*, 1987; McLean *et al.*, 1987). The use of artificial substrates such as L-BAPA and *N*-α-Cbo-D-arginine-glycine-L-arginine-pNA (S-2765) demonstrated that the amino acid specificity for Lp(a) is arginine rather than lysine. A similar specificity for the enzymatic activity associated with the Lp(a) polypeptide has been reported earlier (Jürgens *et al.*, 1977).

The mechanism(s) by which Lp(a) could effect the process of atherosclerosis can, at present, only be speculative. Recently two groups (Hajjar *et al.*, 1989; Miles *et al.*, 1989) reported that Lp(a) inhibits binding of plasminogen to endothelial cells resulting in impaired fibrinolysis. They suggested that this may explain why plasma Lp(a) levels are correlated with the risk for atherosclerosis. Another explanation why Lp(a) is a risk factor which is in accordance with our results is as follows. It has been demonstrated that fibronectin will enhance macrophage uptake of complexes of LDL, heparin and fibrillar collagen (Falcone and Salisbury, 1988). As fibronectin binds heparin (Stathakis and Mosesson, 1977), collagen (Engvall *et al.*, 1978; Ruoslahti and Engvall, 1980) and, as demonstrated here Lp(a), it is conceivable that the uptake of such complexes by macrophages would be markedly enhanced and lead to the intracellular accumulation of Lp(a)-derived cholesteryl esters, apoB and fibronectin—all components of early atherosclerotic lesions.

Fibronectin has been shown to be an early marker for atherosclerotic lesions (Stenman *et al.*, 1980). Thus following cellular damage fibronectin is deposited in damaged tissues and in fibrin clots before tissue repair commences. This could be followed by deposition of Lp(a) and the formation of an early atheromatous plaque. This hypothesis would explain the atherogenecity of Lp(a) and provide a link between thrombus formation and atheroma. Whether the observed decrease in the amount of fibronectin observed during later stages of the atherosclerotic process (Stenman *et al.*, 1980) might be due to the proteolytic activity of Lp(a) remains to be established.

## Materials and methods

### *Lipoprotein(a)*

Plasma from donors with high levels of Lp(a) was collected by plasmapheresis. Individual samples were subjected to ultracentrifugation to obtain the density fraction of 1.05–1.12 g/ml (Havel *et al.*, 1955). This fraction was concentrated by ultra-filtration and chromatographed on a column (2.5 × 100 cm) of Sepharose 4B (Pharmacia, Uppsala, Sweden) (Ehnholm *et al.*, 1971). The fractions containing Lp(a) were pooled and used as such or purified further on lysine–Sepharose as described (Eaton

*et al.*, 1987). To isolate apo(a), the Lp(a) was treated with dithioerythritol (10 mM final concentration) and subjected to density-gradient ultracentrifugation (Fless *et al.*, 1985). The antisera to Lp(a) and apoB have been described previously (Ehnholm *et al.*, 1972). Lp(a) was assayed with a double-antibody immunoradiometric method according to the manufacturer's instructions (Pharmacia). ApoB was assayed with an immunoturbidometric method (Orion Diagnostica, Helsinki, Finland).

### *Fibronectin and its fragments*

Fibronectin was purified from fresh citrated human plasma by a two-step affinity chromatography procedure using gelatin–Sepharose and arginine–Sepharose (Vuento and Vaheri, 1979). The purified protein was dissolved in 0.1 M NaCl, 0.05 M Tris–HCl, pH 7.5, and stored at −70°C at a concentration of 1–3 mg/ml. It was homogeneous by the following criteria: 30 μg of reduced plasma fibronectin migrated in SDS–PAGE as a single polypeptide band of $M_r$ 250 000. In two-dimensional immunoelectrophoresis against antibodies to human plasma proteins (Orion Diagnostica) only one immunoprecipitate identical with that obtained with anti-fibronectin antibodies was obtained. The purified fibronectin did not show any contamination with fibrinogen as judged by immunoelectrophoresis against anti-fibrinogen antiserum (Dako, Copenhagen, Denmark). Fragments of fibronectin were prepared from thermolysin digests using a hydroxylapatite column (Zardi *et al.*, 1985; Borsi *et al.*, 1986). The fragments included a 40 kd gelatin-binding, a 14 kd central heparin-binding, a 110 kd cell-binding, and overlapping C-terminal heparin-binding 29 and 38 kd fragments, (Pande *et al.*, 1987). The polyclonal rabbit anti-fibronectin antibodies have been described previously. These antibodies do not react with fibrinogen (Salonen *et al.*, 1985).

### *Other reagents*

Human t-PA (700 000 IU/mg) was from American Diagnostica, Greenwich, CT, and human u-PA (60 000 IU/mg) from Calbiochem-Behring, La Jolla, CA. Vitronectin was from Telios, La Jolla, CA. Heparin was from Medica, Helsinki, Finland. Human serum albumin and actin were from Sigma, St Louis, MO. Human IgG was purified using Protein A–Sepharose (Pharmacia, Uppsala, Sweden) and bicinchoninic acid (BCA) was from Pierce, Rockford, IL. Phenylmethanesulphonyl fluoride (PMSF), *p*-nitrophenyl-*p*-guanidinobenzoate–HCl (NPGB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) were from Sigma. The chromogenic peptide substrates, Val-Leu-Lys-pNA (S-2251), Pro-Phe-Arg-pNA (S-2302), Val-Phe-Lys-pNA (S-2390), N-α-Cbo-Arg-Gly-Arg-pNA (S-2765) were from Kabi Diagnostica, Stockholm, Sweden. *N*-α-Benzoyl-Arg-pNA (L-BAPA) and tosyl-Arg-methyl ester (L-TAME) were from Boehringer-Mannheim, FRG.

### *Chemical analyses*

Protein was determined by the method of Lowry *et al.* (1951) or using BCA (Smith *et al.*, 1985) with BSA as a standard. Cholesterol was analysed by an enzymatic method (Allain *et al.*, 1974).

### *SDS–PAGE and immunoblotting*

SDS–PAGE was performed on slab gels (Laemmli, 1970). The acrylamide concentration was 3.3% in the stacking gel and 5–16% in the separating gel. The samples were reduced with 10% (v/v) 2-mercaptoethanol in Laemmli's sample buffer. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. Commercially available low mol. wt markers (Pharmacia) were used as standards. Immunoblotting of proteins transferred to nitrocellulose sheets was performed using immunoperoxidase staining according to Towbin *et al.* (1979).

### *Immobilization of proteins onto solid phase*

Purified fibronectin, its fragments, or albumin were immobilized onto flat-bottomed polystyrene wells (Nunc, Roskilde, Denmark) (Salonen and Vaheri, 1979). Duplicate 75 μl samples of fibronectin or albumin (4 μg/ml) or fibronectin fragments (2 μg/ml) were added to each well (corresponding to an active immobilization area of 0.65 $cm^2$) and incubated overnight at room temperature using tight adhesion plastic tape to cover the wells. The immobilization was terminated by incubating the wells with NaCl/$P_i$ containing 0.02% (v/v) Tween 20 (polyoxy-ethylene sorbitan monolaurate), NaCl/$P_i$-Tween. After 10 min at room temperature the wells were washed twice with 200 μl of distilled water and air dried. If not used immediately for binding assays, they were stored at 4°C. The storage had no effect on the binding properties of immobilized fibronectin.

### *Binding experiments and enzyme immunoassay (EIA)*

The procedures used were essentially as described (Salonen *et al.*, 1985). Binding of Lp(a) and apo(a) to immobilized fibronectin or its fragments was performed by adding 75 μl of different concentrations of the purified proteins (up to 10 μg/ml). The incubation solution contained 0.02% (v/v) Tween 20 and 4% (w/v) polyethylene glycol 6000 (PEG, $M_r$ 6000–7500; Fluka, Buchs, Switzerland) (Salonen and Vaheri, 1981). The incubation was done at room temperature for 2 h and was terminated by repeated washings as above.

Fibronectin-bound Lp(a) or apo(a) was determined using a volume of 75 μl of polyclonal rabbit anti-Lp(a) IgG (2.5 μg/ml) in NaCl/$P_i$-Tween containing 4% (w/v) PEG for 1 h at room temperature and washed. This was followed by a 60-min incubation at room temperature with a 1:100 dilution of alkaline-phosphatase-labelled antibodies to rabbit IgG (Orion Diagnostica) in PEG buffer. After washing 0.2% (w/v) disodium *p*-nitrophenylphosphate in diethanolamine–$MgCl_2$ buffer (Orion Diagnostica) was added as substrate and incubated for 15 min at room temperature. The enzyme–substrate reaction was stopped with 1 M NaOH, and the amount of *p*-nitrophenol formed determined spectrophotometrically at 405 nm. In all experiments, either the soluble or solid-phase proteins were replaced, as a control, with BSA or with buffer. When the specific antibodies were replaced with a 20-fold excess of normal rabbit IgG, no reaction occurred.

### *Determination of amidolytic activity*

The amidolytic activity of Lp(a) and apo(a) was determined using chromogenic peptide substrates. The substrates S-2251, S-2302, S-2390, S-2765 and L-BAPA were dissolved in distilled water and L-TAME in 100 mM Tris–HCl buffer, pH 8.0, to 4 mM concentration. In a typical assay, 650 μl of 100 mM Tris–HCl buffer, pH 8.0, and 100 μl of Lp(a) solution were added to a cuvette, mixed and pre-incubated for 5 min at 25°C. To initiate the reaction, 200 μl of substrate was added. The increase in absorbance due to liberated 4-nitroaniline was measured at 405 nm in a Shimadzu UV-260 spectrophotometer. The L-TAME reaction was followed at 247 nm. The results are presented either as absorbance units or as catalytic units (U = μmol/min).

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